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Enhancement of Fern Spore Germination and Gametophyte Growth in Artificial Media

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ABSTRACT.—Spores of bracken fern, *Pteridium aquilinum* were sown in medium with and without agar. No clear trend emerged for germination, but in 14 days, significantly more plants passed from the filamentous to two-dimensional state on agar-solidified medium than in liquid shake-flasks. Spores of *Pteridium aquilinum, Athyrium filix-femina, Dryopteris expansa* and *Anemia phyllitidis* were sown in media containing sucrose. Percentage germination of all four species was significantly enhanced by the inclusion of sucrose.

Fern gametophytes have been described as ideal experimental organisms for use in scientific studies and as model multicellular systems (e.g., Miller, 1968; Hickok *et al.*, 1987). One of the main attractions of these plants is their minimal nutritional requirements, and most researchers have exploited them in cultures employing artificial media. Agar-solidified medium has been the substrate used most routinely for the artificial culture of fern gametophytes, but disadvantages have been reported. Agar can be toxic to plants (e.g., Debergh, 1983), it can perturb sexual expression of ferns (Rubin & Paolillo, 1983), and can slow fern development (Dyer, 1979). Douglas & Sheffield (1992) showed dry weight yield of two species of fern gametophytes, *Pteridium aquilinum* and *Anemia phyllitidis,* to be significantly higher in liquid than in the same medium solidified with agar. This study did not establish, however, whether these higher yields reflected increased germination rates (therefore larger numbers of individuals), higher rates of growth of similar numbers of individuals, or both. Data in Douglas (1994) indicated differences in germination of *Pteridium* and *Anemia* between liquid and agar-solidified medium, with germination percentage significantly higher in liquid medium for both species. Subsequent work in our laboratory using spores from the same collections as used in those experiments, has not repeated these findings. Camloh (1993) found no significant differences in germination of the epiphytic fern *Platycerium bifurcatum* between liquid and agar medium, but reported that "development" was enhanced by liquid medium. The first aim of this investigation was therefore to establish whether liquid medium promotes germination and/or growth of one of the two species used in the study of Douglas and Sheffield, viz. *Pteridium aquilinum* (bracken fern).

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Many workers have included metabolisable sugars in the artificial medium used to germinate fern spores, but no clear trends have emerged from such studies. Hurel-Py (1955) reported that sugars inhibited germination of *Alsophila australis*. Camloh (1993) found that the inclusion of sucrose in liquid medium produced no significant inhibition or stimulation of germination of *P. bifurcatum* spores. Pilot experiments of Douglas (1994) suggested that sucrose greatly enhanced germination of *P. aquilinum* and *A. phyllitidis*. The second aim of this investigation was therefore to establish whether the addition of sucrose enhances or inhibits germination of fern spores. Four species were chosen from different families (*Pteridium aquilinum, Athyrium filix-femina, Dryopteris expansa* and *Anemia phyllitidis*), in an attempt to generate broadly applicable findings.

Materials and Methods

Spores of *Pteridium aquilinum, Athyrium filix-femina, Dryopteris expansa* and *Anemia phyllitidis* were collected and stored (dry in glass vials) at 4°C for either one *(Pteridium),* two (*Athyrium, Dryopteris*) or twelve years *(Anemia).* Spores of the first three taxa were harvested from natural populations of British ferns, and the *Anemia* spores were a kind donation from the glasshouse population of Professor H. Schraudolf (University of Ulm). All spores were sieved through a fine nylon mesh (45μm pore size, Lockertex) to separate them from sporangial debris. They were weighed and kept in plastic vials containing approximately 10ml distilled water with one drop of detergent (wetting agent) at room temperature for 18–24 h. Spores were then surface sterilised with 2.5% (vol/vol) aqueous sodium hypochlorite solution (10–14% available chlorine, BDH) for 2 min, then rinsed three times in sterile distilled water. They were subsequently suspended in sterile 0.5% (w/vol) high viscosity carboxymethyl cellulose (BDH) to allow even distribution of the spores in suspension for inoculation. The basic mineral medium used throughout was that of Moore (1903) adjusted to pH 6.25.

Agar vs Liquid Medium.—Medium containing 1.5gl^{-1} Sigma bacteriological grade agar was dispensed into petri dishes; liquid medium was dispensed as 150ml aliquots into 250ml conical flasks and the flasks sealed with a polyurethane foam bung. Both media were autoclaved at 15lb, 120°C for 15 min.

Liquid Medium With and Without Sucrose.—Medium without sucrose, and medium containing 0.05 and 0.2M sucrose (Sigma) was dispensed into flasks as above, and autoclaved at 10lb, 115°C for 10 min.

Inoculation.—Each spore suspension was adjusted to contain 150,000 (± 500) spores using a Sedgewick Rafter chamber and inoculum added aseptically to each dish or flask to give an initial spore concentration of ca. 1000 spores per ml of medium. The spores were evenly distributed over the agar surface using a flamed glass spreader and excess moisture allowed to evaporate in a laminar flow unit before sealing the dishes with Nescofilm. Flasks were placed on a

rotary shaker at 96rpm and dishes cultured on the bench in the same room, illuminated by daylight fluorescent tubes providing photosynthetically active radiation of 400–700 nm of 106 (±21) $\mu Em^{-2}s^{-1}$ light at the level of the culture surface on a 16h light 8h dark cycle, at 20 (±2)°C.

SAMPLING.—Cultures were sampled after 7 days' incubation in the above conditions for all treatments, and after 14 days for liquid vs agar treatments. Numbers of germinated and non-germinated spores were counted after 7d, and the numbers of individuals that had established 2D growth and those still filamentous after 14d were counted; 250 individuals were counted per replicate.

ANALYSES.—Individual replicates were subjected to analysis of variance, spore germination and 2D growth data were subjected to an independent samples t-test for equality of means, and the means of each different sucrose treatment subjected to chi-square tests to ascertain which sources of variation generated differences between treatments.

RESULTS

AGAR VS LIQUID MEDIUM.—Percentage germination was higher for *Pteridium* spores sown on agar than in liquid medium; the percentage of individuals that had established 2D growth by 14d of culture was considerably higher for both species in agar than in liquid medium (see Table 1). The treatment effects were confirmed when the experiment was repeated, although the overall percentage germination and the percentage variation was not the same for each repeat. Results obtained from spores sown in liquid medium were routinely far more variable than those obtained from agar-solidified cultures.

LIQUID MEDIUM WITH AND WITHOUT SUCROSE.—Percentage germination was very different for different species (see Tables 2–5) and "species tested" was the most highly significant factor accounting for differences between treatments ($F = 139.51$, $p<0.001$, see Table 6). Germination in different concentrations of sucrose was in the order of $0<0.05<0.2$M for all species except *Anemia,* where germination was slightly less in 0.2 than in 0.05M, but both were higher than 0M (see Tables 2–5). The differences between treatments due to concentration of sucrose was significant ($F = 5.88$, $p<0.01$, see Table 6).

DISCUSSION

The results presented here for germination of spores of *Pteridium* sown in liquid vs agar-solidified medium do not mirror those obtained by Douglas (1994). The latter study reported 64(± 4)% germination in liquid, 42(± 4)% in agar, i.e. lower germination on agar than in liquid—the reverse of the findings reported herein. Douglas (1994) found less difference for *Anemia,* but the trend was the same. We could speculate upon an interaction between medium composition and spore age, as our spores of *Pteridium* were only one year old, and those of Douglas were two years old for *Pteridium* and seven years old for

TABLE 1. The mean percentage of *P. aquilinum* spores that germinated, and the mean percentage of individuals that had progressed to 2D growth after 14 d in liquid and agar-solidified Moore's medium (n = 4 for all, s.e. in parentheses).

	Agar	Liquid	P
Germination	85.2 (1.1)	60.0 (16.6)	0.026*
2D growth	94.8 (1.2)	34.2 (13.6)	0.011*

* Less than 0.05, therefore differences between treatments significant

TABLE 2. The mean percentage germination of spores sown in liquid medium containing different concentrations of sucrose for *P. aquilinum* (n = 4).

Sucrose concentration	% germination
0 M	30.4 (8.0)
0.05 M	39.3 (10.2)
0.2 M	43.3 (18.1)

Between concentrations chi-square 145.94 $P < 0.001$

TABLE 3. The mean percentage germination of spores sown in liquid medium containing different concentrations of sucrose for *A. phyllitidis* (n = 4).

Sucrose concentration	% germination
0 M	54.0 (3.1)
0.05 M	57.8 (8.6)
0.2 M	56.4 (8.6)

Between concentrations chi-square 7.54 $P < 0.05$

TABLE 4. The mean percentage germination of spores sown in liquid medium containing different concentrations of sucrose for *D. expansa* (n = 4 for 0M and 0.05M sucrose; n = 3 for 0.2M sucrose).

Sucrose concentration	% germination
0 M	57.8 (3.3)
0.05 M	61.1 (4.6)
0.2 M	66.0 (4.5)

Between concentrations chi-square 33.34 $P < 0.001$

TABLE 5. The mean percentage germination of spores sown in liquid medium containing different concentrations of sucrose for *Athyrium filix-femina* (n = 4 for 0M and 0.05M sucrose; n = 1 for 0.2M sucrose).

Sucrose concentration	% germination
0 M	87.2 (1.3)
0.05 M	91.2 (2.6)
0.2 M	94.7 (na)

Between concentrations chi-square 50.61 $P < 0.001$

TABLE 6. Chi-square tests of differences attributable to the variables examined.

Source of variation:	Chi-square	Probability
Species	4952.35	$P < 0.001$
Concentrations within species	237.45	$P < 0.001$

Anemia, and we obtained higher germination percentages than Douglas overall in our experiments. However, it seems safer to state that there are no predictable differences in germination of *Pteridium* in liquid and agar-solidified medium. This suggests that the higher yields of *P. aquilinum* and *A. phyllitidis* tissue in liquid than in agar-solidified medium observed by Douglas & Sheffield (1992) may have been due to higher growth rates of individuals in medium in liquid form. Our finding that fewer of the individuals growing in liquid medium go on to develop into 2-dimensional plates than on agar medium mirrors the data of Douglas (1994) relating to cell number. The latter study reported significantly fewer cells per gametophyte in 28 day-old individuals of the same species in liquid, than on agar-or agarose-solidified medium. This indicates that although early growth rate is enhanced, subsequent differentiation into the form usually adopted by *Pteridium* is perturbed by liquid medium. This is the reverse of the situation reported for *Platycerium bifurcatum* (Camloh, 1993). This may mean that the response to liquid is species- or ecotype-specific (as *P. bifurcatum* is an epiphytic species, and spores would conceivably land in more aqueous situations on tree bark than in the terrestrial sites colonised by *Pteridium*). Yields of *P. aquilinum* and *A. phyllitidis* are enhanced by using immobilised, rather than shake culture (Douglas & Sheffield, 1992) and perturbation of the development of these two species in liquid can be circumvented by the use of immobilised or air-lift cultures (Sheffield et al., 1997).

Differences in percentages of germinating spores from one population, experiment or culture to another are common in fern experiments (e.g. Pangua et al., 1999), and large differences in overall germination percentage is the rule in simultaneous experiments on different taxa in one set of conditions (e.g. Ranal, 1999). No attempt was made to identify the source of the variation between the species tested herein, because no effort had been made to refine the basic mineral medium or find preferred growth conditions for each fern. It is well known that surface sterilisation reduces percentage germination, and that spore age, storage conditions and culture conditions all have a great influence on fern spore viability (e.g. Miller, 1968; Raghavan, 1989; Camloh, 1993 & 1999; Sheffield, 1996). It is therefore unsurprising that the greatest differences in germination observed were attributable to the taxon tested. However, it is clear that for all four species tested here, whatever the background level of spore viability after collection, storage and surface sterilisation, the addition of sucrose enhances germination in liquid medium (see Fig. 1). This was not reported for *P. bifurcatum* by Camloh (1993), who found no promotive effect of sucrose on germination. Again, therefore, this may reflect species-

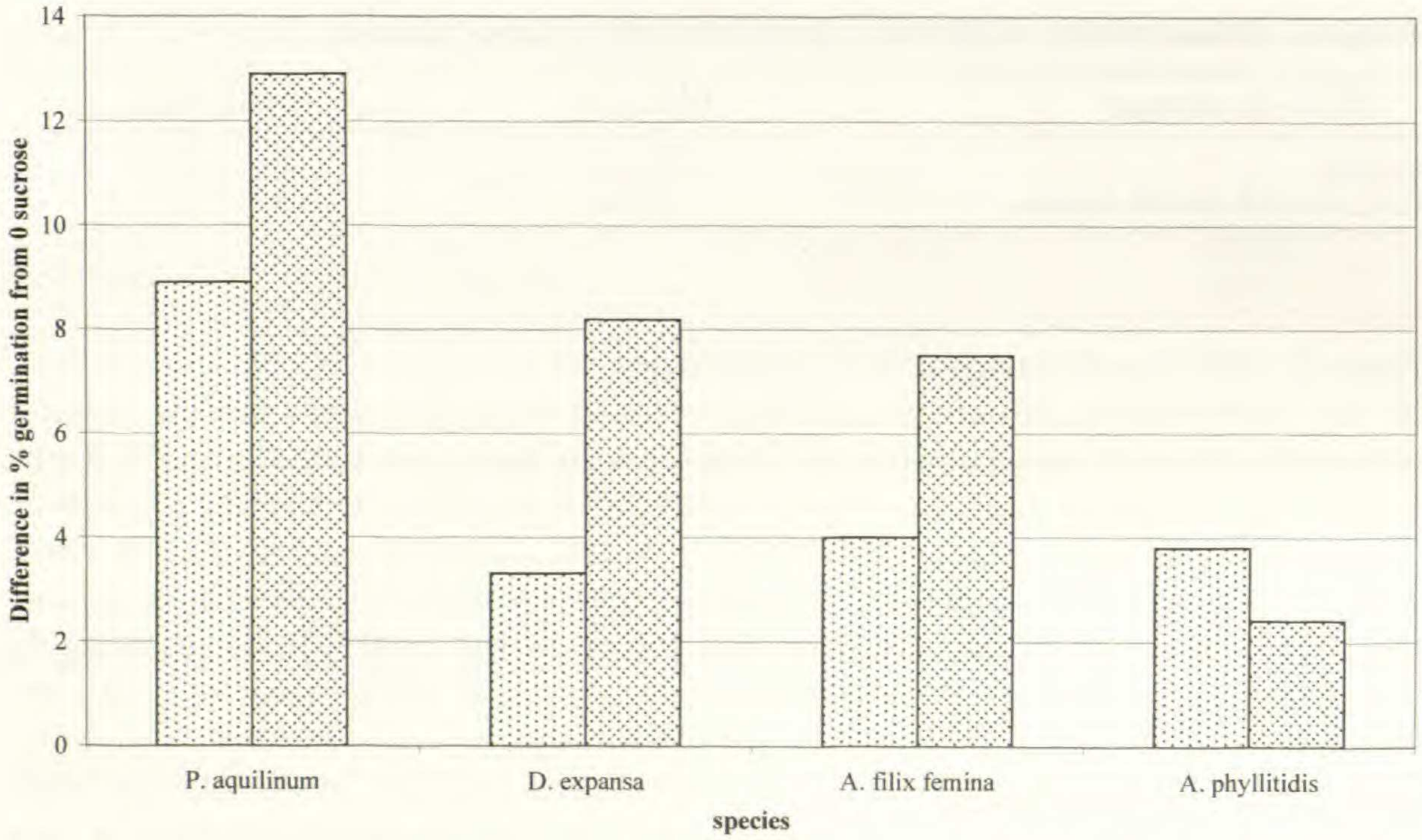

FIG. 1. Histogram of spore germination plotted as % differences between sucrose treatments (0.05M—dotted; 2M—diamond shading) and 0M sucrose controls. Order of species reflects increasing age of spores, youngest far left, oldest far right.

specific processes, or it may again be significant that *Platycerium* is epiphytic, and therefore very different from the terrestrial ferns tested in this study. The spores of *Platycerium* are also much larger than those of the species tested in the present study, so this difference may reflect different levels of storage carbohydrate. All the spores of *Platycerium* tested by Camloh may have had sufficient storage carbohydrate to germinate, in which case no increase in germination with added sucrose would be expected. It may also be that a different sugar or medium component would promote germination in this species. The spores Camloh used were stored before use, and it is known that levels of proteins as well as soluble sugars decline with storage of fern spores (Beri & Bir, 1993). It could be speculated, therefore, that the spores of *Platycerium* that fail to germinate in basal medium including sugar, might germinate if provided with amino acids.

Our finding that sucrose did not promote germination of *Anemia* spores as strongly as the other species may also reflect storage-related decline (see Fig. 1). The *Anemia* spores used here had been stored for many years longer than the other species. A pilot experiment carried out with the same spore sample five years earlier showed far greater promotion with sucrose (Douglas, 1994). In that experiment the germination percentages (s.e. in parentheses, n = 4) were as follows: 0M sucrose 53.1(6.7); 0.05M sucrose 72.5 (6.3); 0.2M sucrose 74.8 (3.4). The percentage germination without sucrose was very similar to that seen here (54.0), but the promotion of germination by sucrose was far more pronounced. This may suggest that during storage another cellular component had declined to limiting levels by the time the spores were retested.

The interaction between promotive effects of sucrose and length of time in storage should clearly be tested for individual spore samples, growth conditions and species. However, the results indicate that sucrose promotes germination in many ferns and would therefore be worth adding to media used to initiate cultures of a previously untested species.

This study examined the effects of sucrose on germination only. Effects of sucrose on subsequent growth of the species studied were not tested and appear to differ from one species to another. Camloh (1993) reported a stimulation of growth (in terms of cell number) using sucrose in medium used to culture *Platycerium,* while Douglas (1994) found evidence of growth inhibition (in terms of numbers achieving 2D growth) in *Anemia* and *Pteridium*. Fernández et al. (1997) reported that sucrose inhibited growth (fresh weight and gemma formation) in gametophytes of *Osmunda regalis.* Further experimentation with a wide range of species may reveal some reliable trends, but the current weight of evidence is on the side of deleterious effects of sucrose on growth of terrestrial species. This, coupled with the enhancement of growth of microbial contaminants which attends the use of media containing sugars, prompts us to suggest a 2-stage cultivation process in order to optimise yield/growth of cultured fern gametophytes. Germination should be effected in media containing sucrose, subsequent growth should employ immobilised or air-lift culture in media with no sucrose.

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